SEROLOGICAL STUDIES OF THE ROOT-NODULE BACTERIA.

II. STRAINS OF RHIZOBIUM TRIFOLII.

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Introduction.

In the first paper of this series (Vincent, 1941) the writer has briefly reviewed some of the earlier work done with the genus *Rhizobium* and described newer methods which enable more information to be obtained as to the antigenic constitution of its members. In the same paper results were presented for strains of *Rhizobium meliloti*; it was found that for no more than six strains was it necessary to postulate a minimum of three flagellar and seven somatic antigens and an examination of forty-two other strains emphasized the heterogeneous nature of the species.

This paper reports results with strains of *Rhizobium trifolii* using the methods which have already been described in detail (loc. cit.).

EXPERIMENTAL.

Organisms used for the Development of Antisera.—These were isolated from several species of *Trifolium*, including some collected from widely separated areas. Details are tabulated below:

Collection No.	Host Plant.	Locality.
46	*Trifolium subterraneum* L.	Dandenong, Vict.
108	*T. subterraneum* L.	Manildra, N.S.W.
36	*T. repens* L.	Manildra, N.S.W.
111	*T. repens* L.	Manildra, N.S.W.
91	*T. glomeratum* L.	Narrandera, N.S.W.
61	*T. tomentosum* L.	Euston, N.S.W.

Isolations were made, the purity of cultures tested and antisera developed as already described in the previous paper.

Cross-agglutination Relationships of Six Strains studied in Detail.

Results of repeated tests of the six organisms used for the development of antisera are set out in Table 1. In each case the figure given takes account of at least two determinations; the agreement between repeated tests was good, as was also that where the position of antigen and antiserum was reversed.

TABLE 1.
Cross-agglutination Relationships of Six Strains.

Testing Antigen.	Sera. *H*-Reaction.						*O*-Reaction.					
	36	108	61	111	91	46	36	108	61	111	91	46
36	*3*	3	3	2	2	—	*4*	4	—	—	—	—
108	3	*3*	3	2	3	—	4	*4*	—	—	—	—
61	3	3	*3*	2	2	—	—	—	*3*	3	—	—
111	3	3	3	*2*	2	—	—	—	3	*3*	—	—
91	3	3	3	2	*2*	—	—	—	—	—	*4*	3
46	—	—	—	—	—	*3*	—	—	—	—	4	*4*

Key: — =no reaction observed at 1/50 final concentration of serum, 1=positive reaction at 1/50, 2=positive reaction at 1/200, 3=positive reaction at 1/800, 4=positive reaction at 1/3,200.

Flagellar Antigens (H-*reaction*).—Two reacting groups were found. Most strains (36, 108, 61, 111 and 91) cross-react to a fair titre, but 46 reacts only with its own antiserum. The *H* titre is generally lower than the *O* (somatic), and in the case of 111 and 91 the serum contains a relatively low concentration of homologous *H* antibody.

Somatic Antigens (O-*reaction*).—As with *Rhizobium meliloti*, the somatic reactions give further differentiation within the groups designated by the flagellar. Three somatic groups can be recognized, viz.:

36 and 108 at least sharing antigen I;
61 and 111 at least sharing antigen II;
91 and 46 at least sharing antigen III.

It is interesting to observe, in the last case, the possession of a common *O* along with different *H* antigens.

Absorption Tests of Antigenic Identity.

Flagellar Antigens.—Strains 36, 108, 61 and 111 were all tested for their ability to absorb antibody of each of the four corresponding sera. Antisera to 36, 108 and 61 were absorbed at 1/50 and tested at final dilutions of 1/100, 1/200, 1/400 and 1/800; 111, because of its low titre, was absorbed at 1/12·5 and tested from 1/25 to 1/200. Antiserum 46 was used to check the validity of the subsequent agglutination test, since it was thought that the viscid nature of the supernatant obtained after exposure to a gummy suspension of antigen might have interfered with the reaction in a non-specific fashion. In every case antiserum to 46 retained its ability to react with its homologous antigen. Strain 91 was tested separately against 36 and 61 and its own serum was exposed to these at 1/25 and tested at from 1/50 to 1/400.

The results of these tests have shown conclusively that each strain is able to absorb completely the others as well as its own antiserum. Table 2 sets out results obtained between 61 and the other cross-reacting strains. *O results* are omitted from the table.

TABLE 2.
Absorption of Flagellar Antibodies between 61 and 36, 108, 111, 91.

Serum.	Absorbed.	Tested.	Result at Successive Dilutions. (i.)	(ii.)	(ii.)	(iv.)	Absorption.
61	Saline	61	H	H	H	(h)	
61	61	61	–	–	–	–	
61	36	61	–	–	–	–	Positive.
61	108	61	–	–	–	–	Positive.
61	111	61	–	–	–	–	Positive.
61	91	61		–	–	–	Positive.
36	Saline	36	H	H	H	h	
36	36	36	(h)	(h)	–	–	
36	61	36	(h)	–	–	–	Positive.
108	Saline.	108	H	H	H	h	
108	108	108	–	–	–	–	
108	61	108	–	–	–	–	Positive.
111	Saline.	111	H	h	h	–	
111	111	111	–	–	–	–	
111	61	111	–	–	–	–	Positive.
91	Saline.	91	H	H	H	H	
91	91	91	–	–	–	–	
91	61	91	–	–	–	–	Positive.

Key: H=full flocculent agglutination, h=slight flocculent agglutination, (h)=very slight but flocculent agglutination.

It is now possible to postulate *A* as a flagellar antigen shared by the strains 36, 108, 61, 111 and 91 and to assign *B* to strain 46.

Somatic Antigens.—Three cross-reacting pairs have been recognized, viz.: 36 and 108, 61 and 111, 91 and 46. Their appropriate cross-absorptions are summarized in

Table 3. The absorption test has been carried out at 1/50 or 1/100 and tested in four steps from 1/100 or 1/200 final dilution.

TABLE 3.
Somatic Cross-absorption Tests.

Serum.	Absorbed.	Tested.	Result at Successive Dilutions. (i.)	(ii.)	(iii.)	(iv.)	Absorption.
36	Saline.	36	+	+	±	−	
36	36	36	−	−	−	−	
36	108	36	−	−	−	−	Positive.
108	Saline.	108	+	+	+	+	
108	108	108	±	−	−	−	
108	36	108	±	−	−	−	Positive.
61	Saline.	61	+	+	+	+	
61	61	61	−	−	−	−	
61	111	61	−	−	−	−	Positive.
111	Saline.	111	+	+	+	+	
111	111	111	−	−	−	−	
111	61	111	−	−	−	−	Positive.
91	Saline.	91	+	+	+	+	
91	91	91	+	−	−	−	
91	46	91	−	−	−	−	Positive.
46	Saline.	46	+	+	+	+	
46	46	46	−	−	−	−	
46	91	46	±	−	−	−	Positive.[1]

Key: + = definite granular agglutination, ± = slight clumping although much less marked than +, − = no reaction.

[1] 46 abs. 91 tested 91 gave a similar result indicating slight residue of 91 antibody.

Antigenic Formulae for Six Strains.

On the basis of these tests it is now possible to put forward the following minimal formulae for the antigenic constitution of the six strains studied in detail:

Strain.	*H.*	*O.*
36	A	I
108	A	I
61	A	II
111	A	II
91	A	III
46	B	III

Agglutination Reactions of Other Cultures of Rhizobium trifolii *against Representative Sera.*

From the formulae outlined directly above it will be seen that it is sufficient to describe *H*-reactions against 36 and 46 and *O*-reactions against 36, 61 and 46. In most cases other sera have been included in the test for confirmation purposes, and always, results with these have supported the relationships already postulated on the basis of cross-agglutination and absorption tests. Results with thirty-two additional strains are summarized in Table 4. Type strains already described are included in the table for completeness.

Examination of these results shows that, even with the restricted number of different sera available for testing, it has been possible to demonstrate a minimum of ten (possibly eleven) distinct serological groups within the thirty-eight strains listed. As in the case of *Rhizobium meliloti* (loc. cit.), the first broad division is on an *H* basis. Most of the strains that gave a flocculent agglutination reacted with the *A* group, only two reacted with *B* and a large number failed to react with either *A* or *B* although adequate motility had been demonstrated in the testing antigen. For these at least another *H*-antigen would have to be postulated. Whilst most of the observed *H*-reactions went to a fair

TABLE 4.
Agglutination Relationship of Various Strains of Rhizobium trifolii.

Collection No.	Host Plant.	Source.	Reactions with Test Sera. Flagellar. 36 A.	Flagellar. 46 B.	Somatic. 36 I.	Somatic. 61 II.	Somatic. 46 III.
36	T.R.	Manildra, N.S.W.	3	—	4	—	—
37	T.S.	Manildra, N.S.W.	3	—	4	—	—
108	T.S.	Manildra, N.S.W.	3	—	4	—	—
109	T.S.	Manildra, N.S.W.	3	—	3	—	—
61	T.T.	Euston, N.S.W.	3	—	—	3	—
34	T.S.	Manildra, N.S.W.	3	—	—	4	—
35	T.S.	Manildra, N.S.W.	3	—	—	4	—
73	T.S.	Beaufort, Vict.	3	—	—	4	—
78	T.S.	Myrniong, Vict.	2	—	—	3	—
42	T.A.	Manildra, N.S.W.	3	—	—	4	—
94	T.F.	Penola, S.Aust.	3	—	—	2	—
111	T.R.	Manildra, N.S.W.	3	—	—	3	—
3*		Wisconsin (No. 238)	2	—	—	2	—
201*	T.S.	Waite, (RT 13)	3	—	—	2	—
91	T.G.	Narrandera, N.S.W.	3	—	—	—	4
153	T.G.	Goonoo Goonoo, N.S.W.	2	—	—	—	4
77	T.S.	Ararat, Vict.	3	—	—	—	—
114	T.S.	Manildra, N.S.W.	2	—	—	—	—
82	T.G.	Wagga Wagga, N.S.W.	3	—	—	—	—
86	T.G.	Temora, N.S.W.	3	—	—	—	—
156	T.G.	Glen Innes, N.S.W.	3	—	—	—	—
160	T.R.	Glen Innes, N.S.W.	3	—	—	—	—
164	T.P.	Glen Innes, N.S.W.	3	—	—	—	—
55	T.G.	(?) Horsham, Vict.	1	—	—	—	—
64	T.R.	Orbost, Vict.	—	3	4	—	—
46	T.S.	Dandenong, Vict.	—	3	—	—	4
22*		Dept. Agric., W.Aust.	—	—	4	—	—
159	T.S.	Glen Innes, N.S.W.	—	—	—	2	1
81	T.R.	Rosedale, Vict.	—	—	—	1	2
4*		Wisconsin (No. 202P).	—	—	—	1	—
39	T.S.	Duntroon, A.C.T.	—	—	—	—	—
157	T.R.	Glen Innes, N.S.W.	—	—	—	—	—
142	T.G.	Goonoo Goonoo, N.S.W.	—	—	—	—	—
145	T.G.	Goonoo Goonoo, N.S.W.	—	—	—	—	—
161	T.P.	Glen Innes, N.S.W.	—	—	—	—	—
165	T.P.	Glen Innes, N.S.W.	—	—	—	—	—
95	T.G.	(?) Temora, N.S.W.	—	—	—	—	—
200*		Waite (RT3, from Wisconsin)	—	—	—	—	—

Key to reactions: —, 1, 3, 4 as for Table 1.

Key to species of host plant: T.A. = *Trifolium arvense* L., T.F. = *T. fragiferum* L., T.G. = *T. glomeratum* L., T.P. = *T. pratense* L., T.R. = *T. repens* L., T.S. = *T. subterraneum* L.

*= Obtained from other collections as indicated. *Wisconsin* = University of Wisconsin; *Waite* = Waite Agricultural Research Institute, Adelaide, S.Aust., per Mr. T. H. Strong.

titre, strain 55 reacts only to low titre with the sera of 36, etc. This kind of reaction is like that which was commonly observed between the two *H* groupings found for strains of *Rhizobium meliloti* and suggests that in the case of this strain there is probably another major *H* antigen.

There are three clearly defined *O* groups within *A* as well as a large group with undetermined *O* and which requires the postulation of at least another somatic antigen. Whilst the four strains reacting with antibody to *I* were all collected from the same

district, it should be noted that three other strains from the same locality fell into the *II* group, and another had an undetermined *O*. The *II* group is the largest defined somatic group within *A*, and included material from widely separated areas and from several species of host plant. Only one strain (153) resembled 91 in having *A* flagellar and *III* somatic antigens; the two, whilst from the same species of host plant, were from widely separated localities.

Strain 64 is the only one which resembled 46 (*B*) in its *H* reaction; its somatic reaction is, however, of type *I*.

Most of the strains which failed to give flagellar reactions with either *A* or *B* also failed to react with any of the somatic antibodies available in the test. These were confirmed as *Rhizobium trifolii* by their ability to produce nodules with clovers. Four strains gave an *O* reaction although in the case of strain 4 it was observed only to a low titre.

There is no evident relationship between host species from which the culture was obtained and the nature of the antigenic constitution of the cell.

Summary.

Methods of serological analysis previously reported for *Rhizobium meliloti* have been applied to strains of *Rhizobium trifolii*.

A detailed study of six strains showed the following relationship:

Strain.	*H.*	*O.*
36 and 108	A	I
61 and 111	A	II
91	A	III
46	B	III

Tests with thirty-two other strains showed that, as with *Rhizobium meliloti*, wider grouping is possible on a flagellar than on a somatic basis; the latter is more strain specific. Twelve strains possessed neither of the *H* antigens postulated, but of these four gave *O* reactions. Eight strains, whilst reacting with the *A* type of flagellar antibody, failed to react with any of the sera for *O*.

At least ten, and probably eleven, groups were recognizable, and of these that reacting with *A*, *II* was the largest clearly defined. This group contained, in all, ten strains collected from five species growing in widely separated areas.

There was no evident relationship between species of host plant and the serological grouping of the strain isolated from it.

Reference.

Vincent, J. M., 1941.—Serological Studies of the Root-nodule Bacteria. i. Strains of *Rhizobium meliloti*. Proc. Linn. Soc. N.S.W., 66, 145-154.